Exhibit C

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Primary CA1 and conditionally immortal MHP36 cell grafts restore conditional discrimination learning and recall in marmosets after excitotoxic lesions of the hippocampal CA1 field

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Summary

Common marmosets (*Callithrix jacchus*, *n* = 18) were trained to discriminate between rewarded and non-rewarded objects (simple discriminations, SDs) and to make conditional discriminations (CDs) when presented sequentially with two different pairs of identical objects signifying reward either in the right or left food well of the Wisconsin General Test Apparatus. After bilateral *N*-methyl-D-aspartate (0.12 M) lesions through the cornu ammonis-1 (CA1) field (7 µl in five sites), marmosets showed profound impairment in recall of CDs but not SDs, and were assigned to lesion only, lesion plus CA1 grafts and lesion plus Maudsley hippocampal cell line, clone 36 (MHP36) grafts groups matched for lesion-induced impairment. Cell suspension grafts (4 µl, 15–25 000 cells/µl) of cells dissected from the CA1 region of foetal brain at embryonic day 94–96, or of conditionally immortalized MHP36 cells, derived from the H-2K^b-tsA58 transgenic mouse neuroepithelium and labelled with [^{3}H]thymidine, were infused at the lesion sites. The lesion plus MHP36 grafts group was injected five times per week with cyclosporin A (10 mg/kg) throughout testing. Lesion, grafted and intact control marmosets (*n* = 4–5/group) were tested on recall of SDs and CDs learned before lesioning and on acquisition of four new CDs over a 6-month period. Lesioned animals were highly impaired in recall and acquisition of CD tasks, but recall of SDs was not significantly disrupted. Both grafted groups of marmosets showed improvement to control level in recall of CDs. They were significantly slower in learning the first new CD task, but mastered the remaining tasks as efficiently as controls and were substantially superior to the lesion-only group. Visualized by Nissl staining, foetal grafts formed clumps of pyramidal-like cells within the denervated CA1 field, or jutted into the lateral ventricles. MHP36 cells, identified by β-galactosidase staining and autoradiography, showed neuronal and astrocytic morphology, and were distributed evenly throughout the CA1 region. The results indicate that MHP36 cell grafts are as functionally effective as foetal grafts and appear to integrate into the host brain in a structurally appropriate manner, showing the capacity to differentiate into both mature neurons and glia, and to develop morphologies appropriate to the site of migration. These findings, which parallel the facilitative effects of foetal and MHP36 grafts in rats with ischaemic CA1 damage, offer encouragement for the development of conditionally immortal neuroepithelial stem cell lines for grafting in conditions of severe amnesia and hippocampal damage following recovery from cardiac arrest or other global ischaemic episodes.

Keywords: marmoset; hippocampal lesions; conditional discriminations; intracerebral transplants; neuroepithelial stem cells

Abbreviations: CA = cornu ammonis; CD = conditional discrimination; MHP36 = Maudsley hippocampal cell line, clone 36; NMDA = *N*-methyl-D-aspartate; SD = simple discrimination

Introduction

The hippocampus, particularly the hilar and cornu ammonis-1 (CA1) fields, is highly susceptible to ischaemic brain injury (Schmidt-Kastner and Freund, 1991) following interruption of cerebral blood flow. Clinical studies and experimental lesions in animals have demonstrated that damage to the hippocampus is associated with deficits in learning and memory. For example, up to 40% of patients recovering from out-of-hospital heart attack have shown moderate to severe memory loss (Roine *et al.*, 1993; Grubb *et al.*, 1996), whilst both neuroimaging and post-mortem studies have revealed selective hippocampal damage, particularly in the CA1 field, in patients with memory impairments following ischaemic episodes (Zola-Morgan *et al.*, 1986; Squire *et al.*, 1990; Kartsounis *et al.*, 1995; Rempel-Clower *et al.*, 1996). Impairments in learning new information (anterograde amnesia) have been most prominent, but recent findings have shown that temporally graded retrograde amnesia may also be evident (Kartsounis *et al.*, 1995; Rempel-Clower *et al.*, 1996).

Global ischaemia is modelled in a controlled and reproducible way by four vessel occlusion in rats in which the vertebral arteries are coagulated and the carotids transiently occluded for periods of 5–30 min (Pulsinelli *et al.*, 1982). Global ischaemia exhibits duration-related trends both in the extent of cell loss and cognitive impairment (Pulsinelli *et al.*, 1982; Nunn and Hodges, 1994; Nelson *et al.*, 1997*a*, *b*), shown chiefly by robust spatial learning and spatial working memory deficits. Although results from correlational studies have been variable (Nunn and Hodges, 1994), recent findings demonstrate that the extent of hippocampal CA1 cell loss induced by four vessel occlusion in rats is related to the extent of the behavioural deficit and confirm that this field is crucially involved in ischaemic learning and memory impairment (Olsen *et al.*, 1994; Nelson *et al.*, 1997*a*, *b*; Block and Schwarz, 1998).

Primate models of global ischaemia, such as constriction of ascending arteries using a neck cuff, combined with hypotension (Zola-Morgan *et al.*, 1992) are more variable in their effects than four vessel occlusion in rats and neurological deficits are seen at durations which produce minimal cognitive impairments (Scheller *et al.*, 1992). Nevertheless, this method has been shown to produce damage to CA1 and CA2 fields and deficits in 'amnesia sensitive' recognition memory tasks, such as delayed non-matching to sample which is as great as that seen in monkeys with hippocampal and parahippocampal cortex lesions. Surgical occlusion of the posterior cerebral artery, which supplies the posterior hippocampus, has also been shown to impair delayed non-matching to sample performance in rhesus monkeys that sustained ischaemic CA1 and CA2 damage (Bachevalier and Mishkin, 1989), although the infarct size was highly variable. Ridley and colleagues (Ridley *et al.*, 1995) demonstrated that *N*-methyl-D-aspartate (NMDA) infused along an angled trajectory resulted in discrete and reproducible lesions of the CA1 field in marmosets, thus providing an alternative method to model effects of ischaemic CA1 cell loss. Cognitive deficits arising from this lesion resembled those shown by animals with damage to septal/diagonal band and fimbria–fornix inputs to the hippocampus, but not by lesions of the entorhinal cortex (Ridley *et al.*, 1995, 1996). These deficits were prominent in visuospatial conditional discrimination tasks (CDs) when different pairs of identical objects determined whether the reward was to be found in the left or right food well. Relatively little impairment was seen in serial spatial reversal, concurrent discrimination or pattern discrimination tasks despite their demands on memory. Ridley and Baker suggest that damage to the hippocampus and fornix impair encoding of information into long-term memory in situations which require simultaneous attention to multiple features of stimuli as well as comparisons with previous stimuli (Ridley and Baker, 1997).

Treatments to alleviate effects of global ischaemic brain damage have typically focused on agents that might interrupt the cascade of events leading to intraneuronal calcium accumulation, the major factor precipitating cell death (Meldrum, 1990), including antagonists at different subtypes of glutamate receptor, calcium and sodium channel blockers, calcium chelators and free radical scavengers (Hunter *et al.*, 1995). Apart from a few exceptions (Block and Schwarz, 1998), significant protection against CA1 cell loss with durations of occlusion above the threshold for cognitive deficit (~10 min with four vessel occlusion in rats) has been difficult to demonstrate. Treatments must usually be given shortly after occlusion and may rescue cells at risk, but they cannot reverse the effects of cell death, which limits their therapeutic potential (Hunter *et al.*, 1995). Use of cerebral transplants to alleviate memory deficits induced by hippocampal damage offers the advantage that the aim of transplanting is to promote functional recovery from damage that has already occurred. Several lines of evidence suggest that foetal hippocampal grafts achieve considerable functional integration into the host brain. Field and colleagues demonstrated that foetal grafts within the hippocampus are contacted by host neurons in an appropriate laminar fashion, provided that cells are homotypically replaced within the lesion site, suggesting that foetal grafts may restore information flow around the hippocampus in a relatively point-to-point manner (Field *et al.*, 1991). This suggestion has been supported by findings that grafts of dentate granule cells, but not CA1 cells, restore long-term potentiation (a correlate of learning) in rats with dentate gyrus lesions (Dawe *et al.*, 1993). In terms of cognitive effects, foetal grafts have shown remarkable specificity. CA1, but not CA3 or dentate granule grafts, improved the performance of rats (subjected to four vessel occlusion) that sustained marked ischaemic CA1 cell loss and showed deficits in a variety of spatial learning and working memory tasks in both a water maze and three-door runway (Netto *et al.*, 1993; Hodges *et al.*, 1996, 1997). The recent findings in marmosets that grafts of

foetal CA1 cells are highly effective in improving recall of conditional tasks learned before lesioning, and also modestly improve CD task learning in animals with profound deficits after CA1 lesions, indicate that cognitive improvement after foetal hippocampal grafts extends to primate species (Ridley *et al.*, 1997). Thus, in terms of eventual clinical application to patients suffering memory loss after ischaemic or traumatic hippocampal damage, where current cerebroprotective drug treatments offer limited therapy, grafting strategies have several advantages: grafts can be accurately targeted to a discrete region of brain damage, their growth can be visualized by neuroimaging techniques with concurrent neuropsychological assessments and, unlike neurodegenerative disease conditions, grafts will not be subjected to the effects of progressive degeneration of the host environment.

Grafts of foetal cells are not likely to provide a viable therapeutic approach for ethical and practical reasons, although they have been important as 'proof of concept' in animal models (Sinden *et al.*, 1995). Several alternative strategies are under development, including genetically engineered cells to deliver neurotrophins or enzymes to specified brain regions, encapsulated cells and cultured stem cells with pluripotent developmental capacity (Gage *et al.*, 1995; McKay, 1997). Three requirements are critical for development of non-foetal donor stem cells. These are (i) the capacity to proliferate in culture but to cease dividing and develop into mature neurons or glia after transplantation into the brain (conditional immortality); (ii) responsiveness to inductive signalling which enables grafted stem cells to differentiate according to the region into which they are implanted (multipotency); and (iii) clonal derivation providing cells of a known lineage in which it is possible to understand and manipulate signals that trigger differentiation along different developmental routes. Recently, Sinden and colleagues have shown that the FGF-2-(fibroblast growth factor-2) responsive Maudsley hippocampal cell line, clone 36 (MHP36), cloned from the E 14 H-2K^b-tsA58 transgenic mouse (Jat *et al.*, 1991) hippocampal neuroepithelium, possesses these characteristics (Sinden *et al.*, 1997). This cell line is conditionally immortalized since the 'immortomouse' expresses the temperature sensitive large T antigen gene in every cell, so that cells derived from this mouse divide at a low (33°C) temperature *in vitro*, but differentiate into mature cells at brain temperature (37–39°C) on implantation. MHP36 cells are multipotent and have been shown to develop into both neurons and glia *in vitro* and *in vivo* (Kershaw *et al.*, 1994; Sinden *et al.*, 1997).

Hippocampal damage following four vessel occlusion or excitotoxic lesions would be expected to provide a favourable environment for grafted MHP36 cells since cell death has been shown to trigger time-dependent changes in the expression of trophic factors, cytokines, neurotrophins and their high affinity receptors (Takeda *et al.*, 1993; Endoh *et al.*, 1994; Maeda *et al.*, 1994) which normally assist in remodelling of circuits following ischaemic or traumatic injury (Lindvall *et al.*, 1994). These factors would be expected to promote site-specific differentiation of grafted cells (Whittemore and White, 1993). Time-course studies of MHP36 grafts in four vessel occlusion ischaemic rats (Sinden *et al.*, 1997) showed that grafted cells labelled with antibodies to β-galactosidase migrated to repopulate the area of CA1 cell loss within 4 weeks of implantation and adopted both neuronal pyramidal cell-like and astrocytic morphologies. This alignment of MHP36 cells within the area of host CA1 cell loss is in marked contrast to foetal cells, which typically mass around the injection site and migrate in clumps along the corpus callosum in rats, rather than seeking the CA1 field. Moreover, ischaemic rats with MHP36 grafts showed substantial recovery of spatial learning and working memory towards control level and performed as well as those with CA1 foetal grafts (Sinden *et al.*, 1997), suggesting a comparable capacity for functional reconstruction of the hippocampus.

The efficacy of MHP36 grafts in rats with CA1 damage prompted us to assess their functional effects in marmosets with CA1 lesions, which had proved responsive to ameliorative effects of foetal grafts on CD task learning. The present experiment therefore aimed to replicate and extend the findings of Ridley and colleagues (Ridley *et al.*, 1997) by comparing lesioned marmosets that had CA1 foetal grafts or MHP36 grafts with lesion-only and intact controls. We aimed to test the recall of simple discriminations (SDs) and CDs after lesioning to establish the lesion deficit and to assign animals of comparable impairment to the three lesion groups. Following transplantation we aimed to assess both long-term recall and the learning of several new discrimination tasks to probe the stability of both lesion-induced deficits and of any graft-induced recovery.

Material and methods

These experiments were carried out in accordance with the UK Scientific Procedures Act, 1986, and guidance notes for good practice issued by the UK Home Office and by the Animal Welfare and Research Advisory Committee of the Institute of Psychiatry.

Animals

Eighteen laboratory-born common marmosets (*Callithrix jacchus*), 10 males and eight females aged between 2 and 5 years and weighing 350–475 g, were housed one to three per cage in a light (lights on 07.30–19.30) and temperature (22–25°C) controlled vivarium. They were fed with mixed fresh fruit and marmoset jelly once a day after training, and marmoset chow was available *ad libitum*. Three times a week they were given pellets made of Cytacon syrup [vitamin B_{12}, Goldshield Pharmaceuticals (Europe) Ltd, Croydon, Surrey, UK] and vitamin D_3 oil (Vetoquinol UK Ltd, Bicester, Oxon, UK) mixed with baby cereal to form a soft dough. This mixture was avidly consumed and small pieces were also used as rewards for most of the animals throughout training.

Behavioural training and testing

Marmosets were trained in the Wisconsin General Test Apparatus to discriminate between objects mounted on disks placed above two food wells. The animal entered a transport box placed in the home cage which was put in front of the screen door of the apparatus. At the start of a trial the screen was raised to reveal the two objects; the monkey reached through the grill of the transport box to touch one of the objects. Displacement of the 'correct' object revealed food underneath, displacement of the 'incorrect' object yielded no reward; the screen was lowered and the objects replaced for the next trial. Typically, animals completed 30–40 trials in a session of 30 min. Animals were trained in two types of task: SDs where one of two objects was always rewarded, and visuospatial CDs using two different pairs of identical objects presented in a pseudorandom order. With one pair the reward was always on the left, with the other pair on the right. All animals were initially trained to a criterion of 90 out of 100 correct trials on SD. This 'shaped' the animals to perform in the apparatus and established a stable performance. The animals were then tested on two examples of the SD task (using new objects for each example) and two examples of the CD task to a criterion of 27 out of 30 correct choices. SD tasks were mastered in ~50 trials and CD tasks in 100–150 trials. Animals were matched for learning ability and assigned to control (n = 5) or lesion (n = 13) groups. After surgery, all animals were tested on retention of one of the SD tasks and one of the CD tasks in order to establish the lesion-induced impairment. In order to qualify for subsequent behavioural analysis, lesioned animals had to have an SD score at or below, and a CD score above their acquisition scores for these two tasks. This would indicate that they did not have a large amount of collateral damage, but they probably did have a substantial bilateral CA1 lesion. Two lesioned animals, additional to the 13 that completed the experiment, were excluded because they were not impaired on the CD task. The remaining lesioned animals were matched for lesion induced learning impairment and allocated to three groups: lesion only (n = 5), lesion plus CA1 grafts (n = 4), lesion plus MHP36 grafts (n = 4). Following grafting, animals were tested for retention of another SD and CD task learned before lesioning. They were then tested with four new CD tasks over a 6-month period. Animals which did not achieve criterion in 300 trials were deemed to have 'failed' and moved to the next task.

Surgery

Animals were operated in batches of 2–3 so that groups of lesion and lesion plus grafts were tested simultaneously. Control marmosets were not operated. For lesioning, marmosets were pre-treated with dexamethasone [2.0 mg/kg, i.m. (intramuscular); Merck, Sharp and Dohme Ltd, Harlow, UK] to reduce oedema, and anaesthetized with alphaxalone-alphadolone (Saffan, 18 mg/kg, i.m.; Schering-Plough Ltd, UK). Stereotaxic co-ordinates were set up to allow the angled penetration of a 10 µl Hamilton syringe through the CA1 field via a burr hole drilled in the occiput, as described by Ridley and colleagues (Ridley *et al.*, 1995). Five equidistant infusions of 0.7 µl of NMDA (0.12 M in 0.9% saline, pH adjusted to 7.4 with 1 N NaOH) were made through this trajectory. Following surgery animals were monitored for body temperature for several hours until conscious and feeding or drinking had been observed. Marmosets remained in an incubator overnight, with food and water available. They were then housed singly in post-operative cages until normal feeding and activity were apparent, and then returned to their home cages. About a week after surgery lesioned animals were tested with one SD and one CD task learned pre-operatively. Animals assigned to transplant groups then received cell suspension grafts along the same sites as the lesion, following the same procedure, ~3 weeks after lesioning.

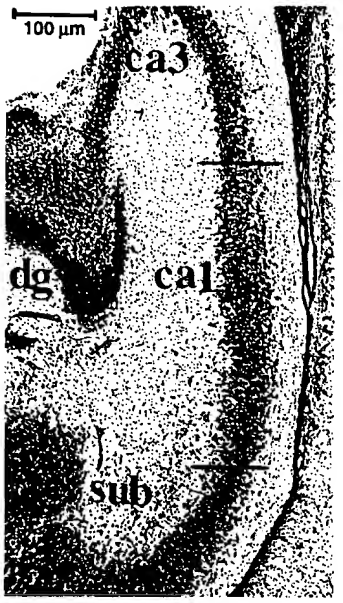


Fig. 1 Nissl stained coronal section through foetal (embryonic day 94–96) hippocampus showing approximate lines of dissection of the CA1 field. At this posterior level and stage of development the pyramidal cell layer is narrow and densely packed relative to the adult CA1 field (cf. Fig. 5A). dg = dentate gyrus; sub = sabiculam; scale bar = 100 µm.

For foetal grafts, twin or triplet embryos (3.5–4 cm crown–rump length) were removed at embryonic day 94–96 (calculated as 106–108 days after previous parturition) by laparotomy and the mother was stitched and placed in an incubator for recovery. Foetal heads were removed, and placed in tubes containing 1 mM *N*-acetyl-L-cysteine (Sigma, Poole, Dorset, UK) in Hanks' balanced salt solution (Sigma), packed in ice and transferred to the home laboratory of the marmosets awaiting surgery (~3 h). Brains were then dissected on ice using an operating microscope. The CA1 field of the hippocampus was removed (Fig. 1) and cut into fragments, combining tissue from all available foetuses, since each recipient received all the CA1 tissue from one donor. Tissue

was incubated for 20 min at 37°C in Hanks' balanced salt solution containing 0.1% trypsin (Sigma) and 0.01% DNAase (Sigma) and 1 mM *N*-acetyl-L-cysteine. Digestion was stopped by replacing the supernatant with Hanks' balanced salt solution/*N*-acetyl-L-cysteine containing 0.01% soybean trypsin inhibitor and 0.06% bovine serum albumin, fraction V (Sigma) for 10 min at room temperature. Tissue was washed several times in the Hanks balanced salt solution/*N*-acetyl-L-cysteine medium and dissociated into a cell suspension by gentle movement through a small bore fire polished pipette. Viable cell density was counted before and after transplantation by trypan blue exclusion in a haemocytometer. The final suspension (50 µl) contained 15 000–23 000 cells/µl. Initial viability was ~95% and fell to ~80% after transplant surgery. Cells were injected at five points along the lesion trajectory bilaterally (4 µl/site) using the same procedure as for lesioning.

The derivation of MHP36 cells has been described by Sinden and colleagues (Sinden *et al.*, 1997). Cells for grafting were brought up from frozen stock (passage 36) and suspended at a concentration of 25 000 cells/µl in 1 mM *N*-acetyl-L-cysteine in Hanks' balanced salt solution. Two days before transplantation cells were pulsed with 0.5 µCi/ml [^{3}H]thymidine to permit identification by autoradiography. This was necessary because expression of the β-galactosidase marker has been found to be low after ~6 weeks of survival, and therefore this label may not be optimal for long-term behavioural studies. As with foetal grafts, MHP36 cells were suspended in Hanks' balanced salt solution/*N*-acetyl-L-cysteine and injected at five sites (4 µl/site, 24–25 000 cells/µl) along the lesion trajectory. Cell viability was 98% before transplantation and 85% by the end of surgery. Grafted marmosets received i.m. injections of cyclosporin A (Sandimmun, Sandoz Ltd, Frimley, Surrey, UK), 10 mg/kg suspended in Cremophor EL (Sigma) five times a week until sacrifice.

Testing commenced 12 weeks after grafting in animals with foetal grafts, and 6 weeks after grafting in animals with MHP36 grafts, following evidence from previous time-course studies in rodents of the integration of grafted foetal (Mudrick and Baimbridge, 1991) and MHP36 (Sinden *et al.*, 1997) cells into damaged hippocampus. Lesioned and non-lesioned controls were tested along with the grafted animals. Table 1 gives the approximate time-course of the experiments, but it should be noted that animals were run in overlapping batches of two to four from different groups and therefore not during the same period of time.

Table 1 *Duration of the phases of the experiment*

Weeks (approx.)	Procedures
1–8	Phase 1: acquisition of SDs and CDs
9	Lesion surgery
11–12	Phase 2: post-operative retention of SDs and CDs
13	Transplant surgery
19	Phase 3: post-transplant retention of SDs and CDs in marmosets with MHP36 grafts
21–31	Phase 4: acquisition of new CDs in marmosets with MHP36 grafts
25	Phase 3: post-transplant retention of SDs and CDs in marmosets with CA1 grafts
26–41	Phase 4: acquisition of new CDs in marmosets with CA1 grafts

Animals were run in batches of 2–4 marmosets from different groups at staggered intervals, but all animals followed this approximate time-course. The overall length of time for each phase was determined by the slowest animals in each batch.

Histology

At the end of behavioural testing animals were perfused with 4% paraformaldehyde (Sigma) and the brains removed for wax embedding. Sections of 7 µm were cut through the hippocampus and serial sections taken for Nissl and glial fibrillary acidic protein staining, to identify graft masses and pyramidal or astrocyte-type cells within grafts, and host glial reactivity to lesion damage and the presence of grafts. Sections from brains with MHP36 grafted cells were additionally treated for autoradiography and immunoreactivity to β-galactosidase and βIII-tubulin (monoclonal anti-β-tubulin isotype III, Sigma) to identify labelled grafted cells and whether they expressed neuronal characteristics.

Data analysis

The number of trials to criterion (maximum 300) in the SD and CD tasks was compared across groups by two-factor (group × task) analyses of variance (Genstat V PC) for acquisition and each retention phase (phases 1–3). A repeated measures analysis was used for comparison across groups for the acquisition of four new CD tasks after transplantation (phase 4). Grafted groups were compared with lesioned and intact controls by Newman–Keuls comparison of means, and orthogonal trends were used to detect systematic changes in performance in phase 4.

Results

Acquisition of SDs and CDs (phase 1)

There were no differences between groups in the mean number of trials required to learn SDs (~40–50) or CDs (100–150). All marmosets found SD tasks easier to learn than CD tasks [$F(1,14) = 25.53$, $P < 0.001$] so there were no interactions between groups and task (Fig. 2, phase 1).

Post-lesion retention of SDs and CDs (phase 2)

All lesioned animals were substantially impaired in retention of CDs but not SDs. Lesioned groups differed from controls ($P < 0.01$) for the CD but not the SD task. Marmosets assigned to the lesion-only and the two grafted groups did not differ from each other in the extent of impairment in recall of the CD task (Fig. 2, phase 2). This selective

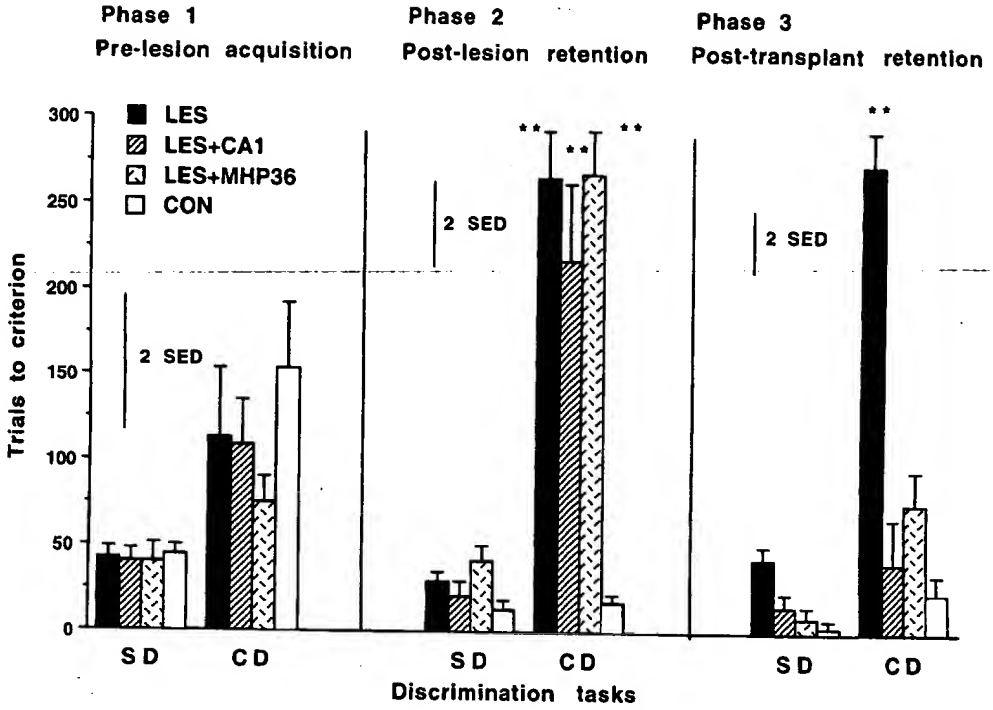


Fig. 2 Mean (±SEM) number of trials to criterion (27 out of 30 correct choices) in SDs and CDs in phases 1–3. Phase 1: pooled results for learning of two tasks before lesioning showing that monkeys in all groups required a similar number of learning trials (~50 for SDs, 100–150 for CDs). Phase 2: retention of one of these tasks in control and lesioned marmosets. Note the selective and substantial lesion deficit in the CD task relative to non-lesioned controls (CON). Marmosets assigned to lesion-only (LES), lesion plus CA1 foetal grafts (LES + CA1) and lesion plus MHP36 grafts (LES + MHP36) groups did not differ in the extent of lesion-induced impairment. Phase 3: post-graft retention of a second SD and CD task learned before lesioning. Lesion-only marmosets continued to show a substantial deficit in recall of the CD task but not the SD task relative to controls. Lesioned marmosets with foetal or MHP36 grafts did not differ from controls in the number of trials to criterion in the CD task despite their substantial impairment before transplantation. SED bars show twice the standard error for the difference in means between groups. Difference from controls, $^{**}P < 0.01$.

lesion-induced CD deficit was shown by substantial differences between groups [$F(3,14) = 26.08$, $P < 0.001$] and the highly significant interaction between groups and task [$F(3,14) = 22.22$, $P < 0.001$]. The difference between tasks seen in acquisition was magnified by the lesion-induced CD impairment [$F(1,14) = 179.99$, $P < 0.001$].

Post-transplant retention of SDs and CDs (phase 3)

After transplantation, groups with CA1 and MHP36 grafts recalled CDs as efficiently as controls, but the lesion-only group remained substantially impaired relative to controls and both of the grafted groups ($P < 0.01$ in all comparisons; Fig. 2, phase 3). The lesion-only group also required more trials to criterion for the SD task than controls, but this difference was not significant. As a result of the continued CD impairment in lesion-only animals, there were marked differences between groups [$F(3,14) = 61.79$, $P < 0.001$] and a substantial group × task interaction [$F(3,14) = 34.04$, $P < 0.001$]. As in post-lesion retention, the difference between tasks was highly significant [$F(1,14) = 103.08$, $P < 0.001$].

Acquisition of CDs after transplantation (phase 4)

Learning scores (trials to criterion) of lesioned groups with and without grafts and the control group were compared in four new CD tasks over a period of 5–6 months after transplantation (Fig. 3). There were substantial differences between groups [$F(3,14) = 30.85$, $P < 0.001$] and a significant linear trend of improvement over tasks [$F_{lin}(1,40) = 27.54$, $P < 0.001$; Fig. 3]. On the first task all the lesioned groups were impaired relative to controls ($P < 0.01$). However, the lesion-only group was also significantly more impaired than each of the grafted groups ($P < 0.01$). Performance of the grafted groups was thus intermediate between control and lesion level at this point. On the subsequent CD acquisition tasks (CDs 2–4) both the lesion plus CA1 grafts and the lesion plus MHP36 grafts groups improved to control level, whilst the lesion-only group continued to show profound impairment. Thus, the lesion group differed substantially from the control and both of the grafted groups throughout acquisition of CDs 2–4 ($P < 0.01$) and there was no evidence for a reduction in the lesion deficit during the training period.

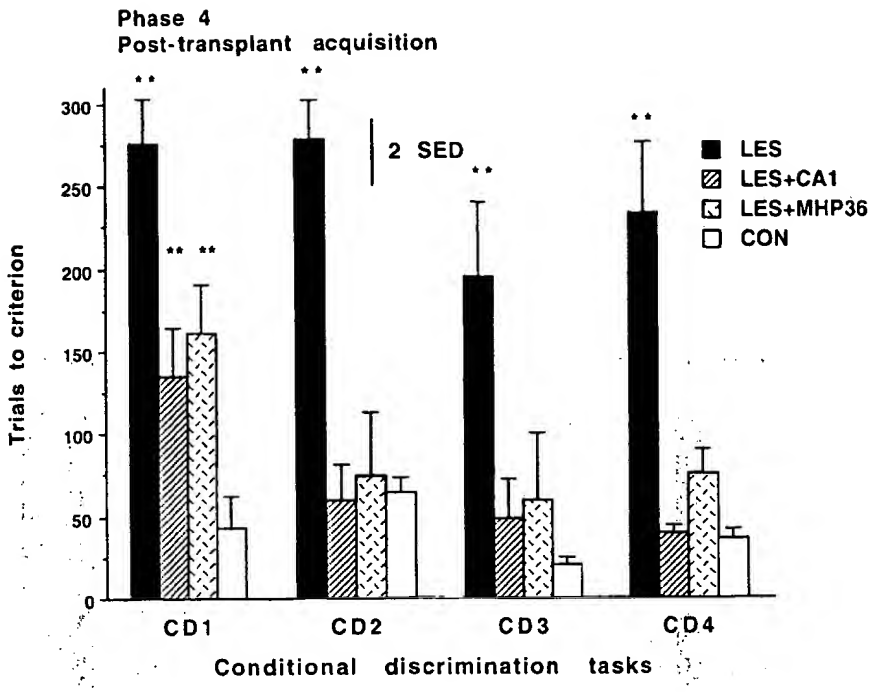


Fig. 3 Mean (±SEM) number of trials to criterion (27 out of 30 correct choices) during acquisition of four new CD tasks in phase 4. The lesion control group remained substantially impaired relative to control and grafted groups throughout training. Marmosets with CA1 or MHP36 grafts were also impaired relative to controls in the first CD task, although they learned significantly more rapidly than the lesioned controls. For CD tasks 2–4 neither grafted group showed a learning impairment. Groups as in Fig. 2. SED bar shows twice the standard error for the difference in means between groups over the repeated measures for tasks. Difference from controls, $^{**}P < 0.01$.

Histological assessment

Lesions

In all animals lesions were associated with a shrinkage of the hippocampus and marked enlargement of the lateral ventricles (Fig. 4B and C). Lesion sites were characterized by loss of CA1 pyramidal cells and marked glial reactivity (Fig. 4C), shown by dense glial fibrillary acidic protein staining within the lateral hippocampus (CA1 field) and the cortical margins of the lateral ventricles. This reactivity was comparable in lesioned and grafted animals, and therefore grafts do not appear to have modified activated glial response to neurotoxic injections.

Loss of hippocampal cells was assessed in Nissl stained coronal sections in lesioned and control animals by cell counts at level AP (anterior–posterior) 4.5 mm (Stephan *et al.*, 1980) at the centre of the lesion axis. CA1 cell counts were carried out within a field measuring 0.62 mm^2, which was placed over the CA1 area. It was possible to exclude transplanted cells from the counted cells for several reasons. Foetal cells in all but one case seeded outside the CA1 sector used for counting. In the one animal with grafted cells within this field, the graft structure was clearly delineated and grafted cells differed in organization and density from normal host CA1 cells. The grafted murine MHP36 cells in the field were much smaller than marmoset CA1 cells, and as only the large CA1 pyramidal cells were counted, small grafted cells, with nuclei only half the diameter of host CA1 cells, were excluded. Counts showed that bilateral numbers of CA1 cells, averaging ~300 in controls, fell to means ranging from 9 (lesion plus MHP36 grafts) to 56 (lesion plus CA1 grafts) in the lesioned groups, a difference that was highly significant [$F(3,14) = 25.45$, $P < 0.0001$; Table 2]. Mean differences between controls and the three lesioned groups were robust ($P < 0.001$), whereas none of the lesioned groups differed significantly from each other. Lesion sites at AP 4.5 mm, shown in Fig. 5, provide sections with the least and most extensive CA1 cell loss across all animals within in each group. In most lesioned animals there was extensive (80–90%) bilateral cell loss through the anterior–posterior axis of the CA1 field. In one monkey with lesion-only and two with CA1 grafts, a high proportion of cells survived on one side (i.e. 50–80% of control level at AP 4.5 mm; Fig. 5), but very few CA1 cells were detected on the other side. In monkeys with MHP36 grafts the lesions almost totally obliterated pyramidal cells throughout the CA1 field. Thus, the functional recovery in grafted animals cannot be attributed to the chance occurrence of less severe lesions than in animals with lesions alone. In addition to CA1 cell loss, four animals (two from the lesion plus CA1 grafts group and one from each of the lesion plus MHP36 grafts and lesion-only groups) showed unilateral damage to the dentate gyrus, and in three animals (two from the lesion plus MHP36 grafts group and one lesion-only animal) damage extended unilaterally into the subiculum. The two animals that were excluded on behavioural grounds (see Material and methods) were found, on histological examination, to have sustained only unilateral CA1 lesions.

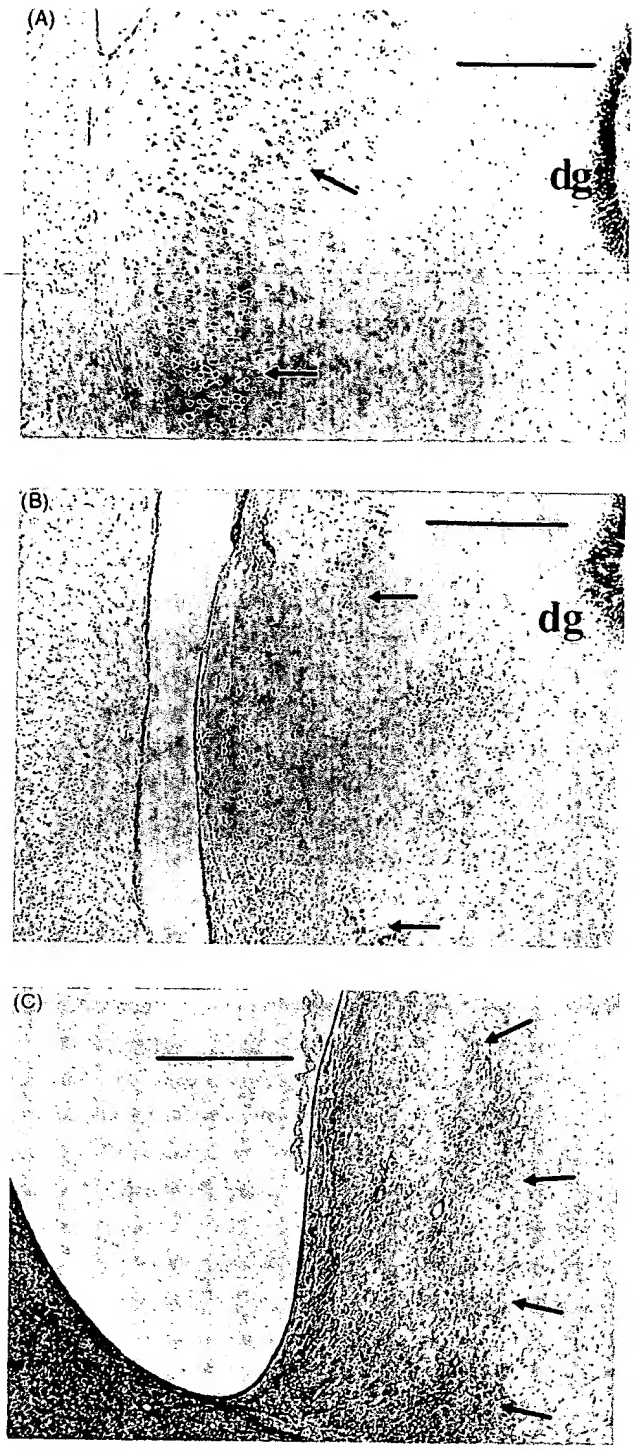


Fig. 4 Coronal sections through control and hippocampal lesioned brain. (**A**) Nissl stained section from a non-lesioned control showing the broad and even distribution of CA1 pyramidal cells (arrows). (**B**) Nissl stained section from a lesioned marmoset showing marked reduction of CA1 pyramidal cells from the CA1/CA3 junction to the start of the ventral curvature where the CA1 merges with the subiculum (arrows). (**C**) Section stained for reactivity to glial fibrillary acidic protein. Marked glial response (arrows) is seen clearly delineating the lesion site. This occurred in all lesioned animals whether grafted or not. All three sections were taken ~3.5–4 mm anterior to the interaural line. Note the enlargement of ventricles in lesioned animals (**B** and especially **C**) relative to the control (**A**). dg = dentate gyrus. Scale bar = 500 μm.

Table 2 *Mean (± SEM) CA1 cell counts at AP 4.5 mm before the inter-aural line in control, lesioned and grafted marmosets*

Group	Number of cells	Difference from controls (*P*)
Control	297.0 ± 9.0	–
Lesion	23.5 ± 20.6	<0.001
Lesion plus CA1 grafts	56.5 ± 32.9	<0.001
Lesion plus MHP36 grafts	8.9 ± 7.1	<0.001

Foetal grafts

Nissl stained sections clearly revealed grafted cells of CA1-like morphology in all four animals that received transplants, when examined ~8 months after grafting. Foetal grafts were of two types: those that formed dense swirls within the lesioned CA1 field (Fig. 6) and those that formed lobular masses attached to the edge of the CA1 field (Fig. 7), jutting into the enlarged ventricular space and, in some cases, forming an artificial bridge between the hippocampus and the white matter adjacent to the temporal cortex (Fig. 7). In one animal grafts on both sides were of the lobular type, sited at anterior levels (~4.5–6.5 mm anterior to the inter-aural line). In the second marmoset grafts were seen within central and posterior regions of the hippocampal lesion site (~1.5–4.5 mm anterior to the inter-aural line). In the third animal a ventricular graft was sited in an anterior position and a smaller intrahippocampal graft was located at a posterior site (~1.5–2.5 mm anterior to the inter-aural line). In the fourth animal the right hippocampus was damaged during processing so that, although there appeared to be grafted cells on the edge of the damaged CA1 field, these could not be clearly identified. This animal had an extensive anterior lobular graft (~4.0–6.5 mm anterior to the inter-aural line) attached to the left hippocampus. Since improvement in the recall and learning of CDs was not significantly different from controls in all animals with foetal grafts in all CD tasks except one, these grafts seemed to be adequate to produce maximal recovery.

MHP36 grafts

MHP36 cells, examined ~6 months after grafting, were also clearly evident in Nissl stained sections in all four animals (Fig. 8). MHP36 grafts were of a consistent appearance, which differed from the two types of foetal graft, and exhibited two characteristic features. First, MHP36 cells formed diffuse aggregates around the site of injection. These aggregates were less compact than the tight swirls formed by foetal cells within the hippocampus and more integrated with the host tissue (cf. Figs 6 and 8B). Secondly, MHP36 cells migrated away from the site of transplantation and were found positioned throughout the lesioned CA1 field. MHP36 cells identified by antibodies to β-galactosidase (Figs 8 and 9A) and by autoradiography (Fig. 9B and C) were dispersed widely in the pyramidal CA1 field and the hippocampal

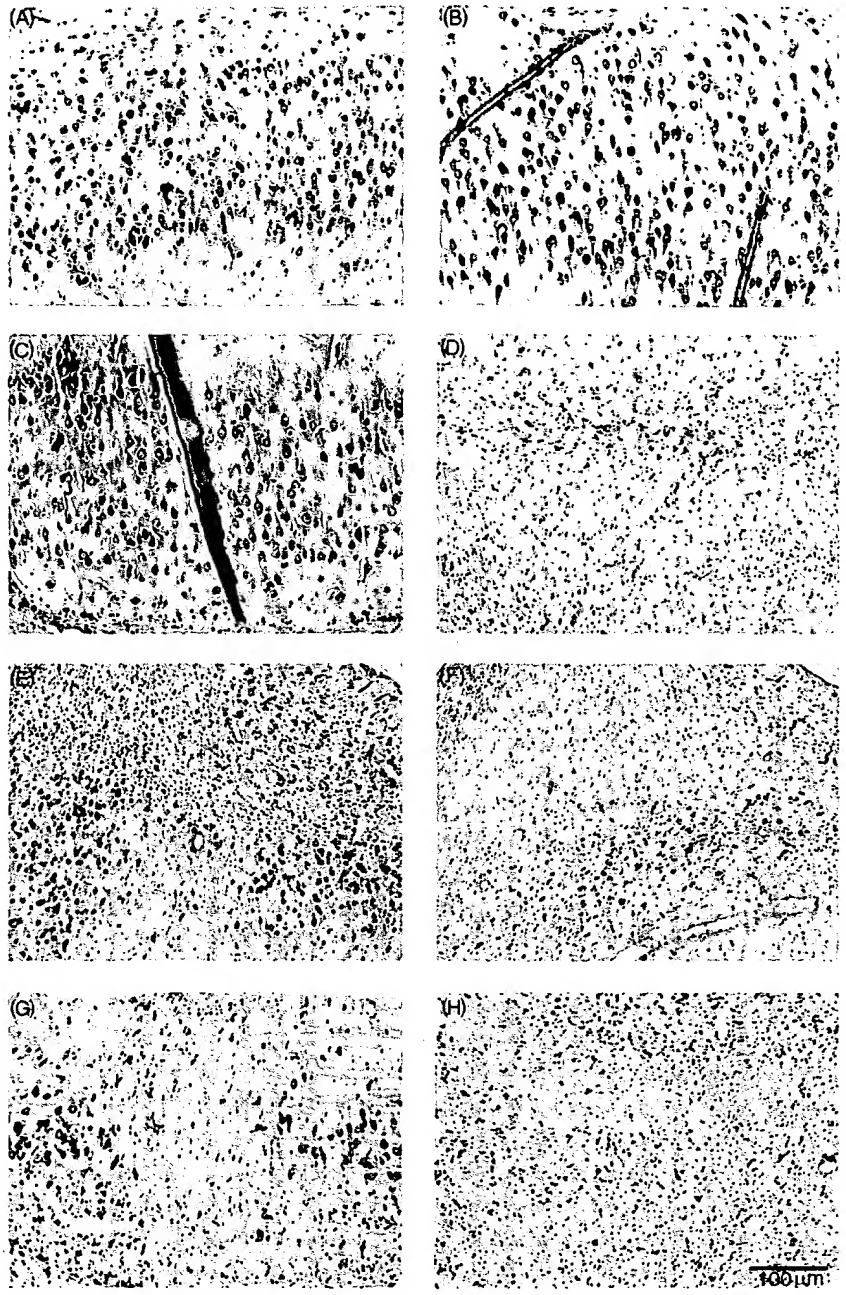


Fig. 5 CA1 cells in control and lesioned marmosets. Sections at AP 4.5 mm (Stephan *et al.*, 1980) in the CA1 area used for counting cells (see Table 2). Sections on the left (**A**, **C**, **E** and **G**) show the greatest number of surviving host pyramidal cells and those on the right (**B**, **D**, **F** and **H**) show the smallest number of pyramidal cells from either hemisphere across all animals within each group. In controls (**A** and **B**) cell counts averaged 300 (range 254–327). One marmoset in the lesion-only (**C** and **D**) and two marmosets in the lesion-plus-CA1-grafted groups (**E** and **F**) showed good cell survival (to 50–80% of control level) in the hippocampus on one side of the brain, though in each case there were few or no surviving cells in the contralateral hippocampus. In marmosets with MHP36 grafts lesioning was almost complete; the maximum number of surviving pyramidal cells counted was 68. Within each of the lesion groups a zero score was recorded for at least half of the sections at this level. Scale bar = 100 µm.

fissure. Other grafted MHP36 cells were found to have migrated into the adjacent white matter of the temporal cortex. In one animal that exhibited some dentate gyrus damage, β-galactosidase labelled MHP36 cells were seen in the dentate granule layer.

Two approaches were used to identify individual cell

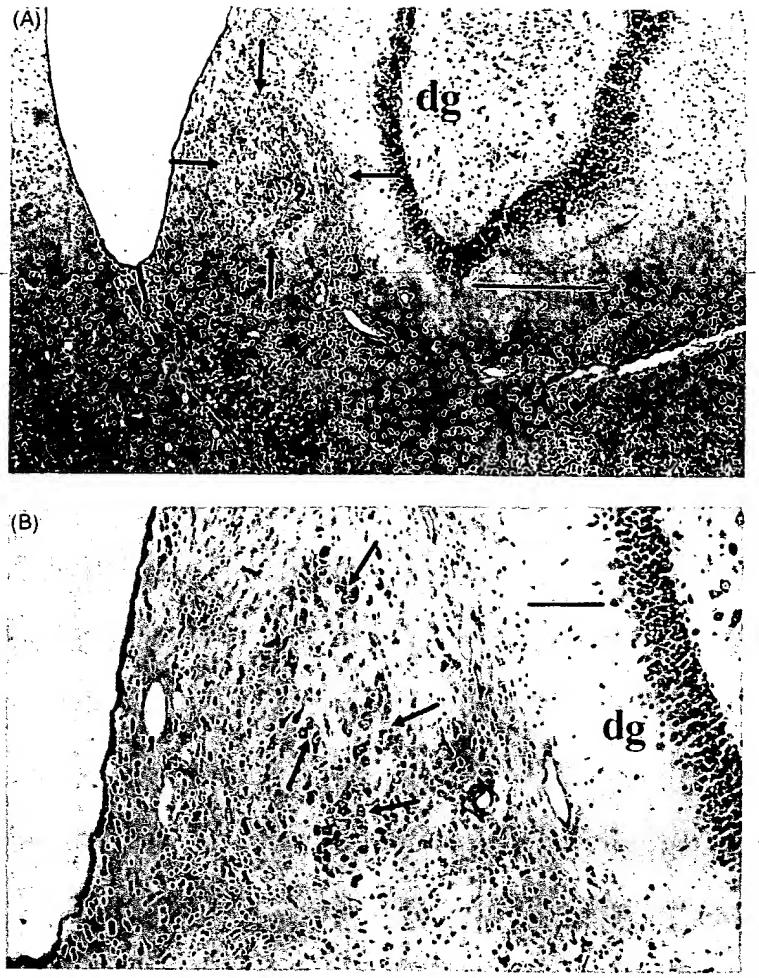


Fig. 6 Nissl-stained coronal sections showing a cluster of grafted foetal CA1 cells (arrows) within the lesioned host CA1 field. (**A**) Scale bar = 500 μm. (**B**) At higher magnification (×100) normal looking pyramidal cells (arrows) can be seen in the graft mass. dg = dentate gyrus; scale bar = 100·μm.

phenotypes of MHP36 grafts. First, serial sections were stained for β-galactosidase and βIII-tubulin to identify neuronal phenotypes. The grafted areas proved positive for both β-galactosidase and β-tubulin indicating that some grafted cells express a neuronal phenotype. The same area stained positive for glial fibrillary acidic protein, but this was also seen in marmosets with lesions alone (Fig. 5C) and with foetal grafts, indicative of lesion-induced activated host astrocyte invasion. Secondly, examination of β-galactosidase labelled cells indicated differential morphology. Cells resembling classical neuronal or astrocytic phenotypes were seen near the transplantation sites (Fig. 8B). Cells that migrated away from the transplantation site and integrated into the CA1 field showed either a pyramidal or an astrocytic morphology (Fig. 9A). Cells which migrated into the white matter appeared to be exclusively astrocytic. In an animal with MHP36 cells in the dentate granule layer, the cells appeared to be solely neuronal and of granular appearance. Photomicrographs of graft histology can be viewed in colour at the ReNeuron web site (http://www.reneuron.com/).

Discussion

Lesion effects

The results showed that bilateral NMDA lesions of the CA1 field resulted in stable and long-lasting impairment both of retention of CDs first learned before lesioning, and of the ability to learn new discriminations. The finding that recall of SDs was not significantly disrupted suggests that the lesion induces a very specific cognitive impairment rather than having an effect on perception, motivation or motor function. Ridley and colleagues (Ridley *et al.*, 1995, 1997) have shown that CD tasks are disrupted by medial septal/diagonal band and fimbria–fornix lesions, as well as by intrahippocampal damage, but not by entorhinal cortex lesions. The present results confirm these findings of a substantial selective disruption of acquisition and recall of CDs following damage within the hippocampus, and indicate that this disruption persists through a series of new acquisition tasks so that it may, in effect, be irreversible by time or practice. The only circumstances in which monkeys with hippocampal damage are unimpaired in CD tasks are (i) in retention of tasks

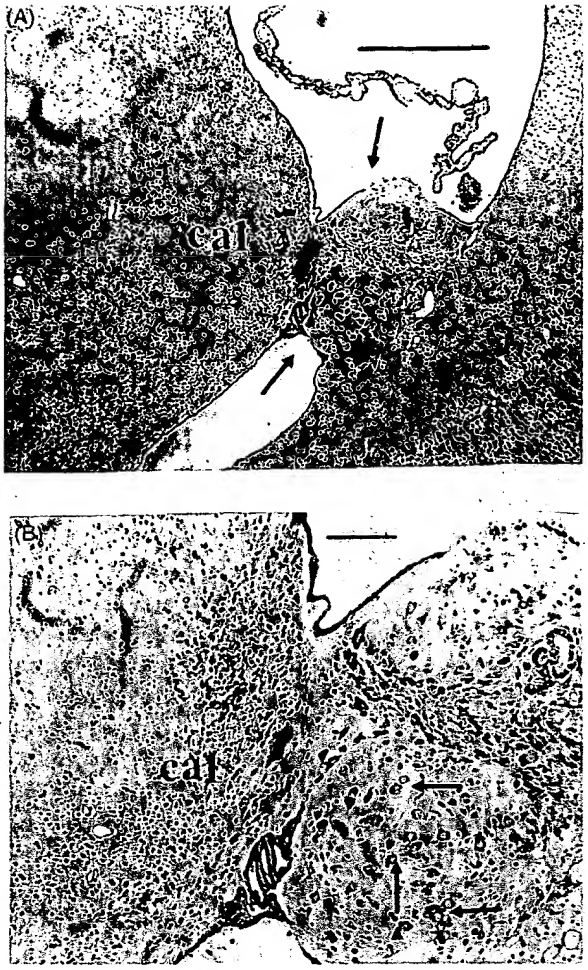


Fig. 7 Nissl stained coronal sections showing a lobular foetal CA1 graft mass attached to the ventricular margin of the lesioned CA1 field. (**A**) The graft formed an artificial bridge to the white matter bordering the temporal cortex across the lateral ventricle. Scale bar = 500 μm. (**B**) At higher power magnification (×100) healthy looking grafted pyramidal cells (arrows) can be seen within the graft mass. Scale bar = 100 μm.

learned (very slowly) in the lesioned state; and (ii) with repeated runs of the same stimuli during training (rather than a pseudorandom schedule), which may encourage learning of the task as separate SDs, rather than learning the conditional rule (Ridley *et al.*, 1996, 1997). Ridley and Baker argue that CD tasks require the ability to process different possibilities about the same stimuli simultaneously, rather than learning single invariant relationships serially (Ridley and Baker, 1997), conceptualized by Perner (Perner, 1991) as 'multi-modal' as opposed to 'single up-dating' information processing. This interpretation is consistent with previous suggestions that the hippocampus acts as a comparator specialized for processing 'relational' information (for a review, see Eichenbaum *et al.*, 1995). Findings that monkeys with septohippocampal system damage can only master CD tasks laboriously, indicate that this task normally requires a specialized type of encoding process for storage of information in long-term memory, for which the hippocampus is critically important. Our findings indicate that CDs learned by the intact brain also require the hippocampus for retrieval (cf. Squire and Alvarez, 1995). However, a clear implication of graft-induced reversal of retention deficits is that information is not stored long-term within the hippocampus. Hippocampal-dependent CDs appear to be highly suitable for the investigation of graft effects following hippocampal damage, because they provide stable levels of impairment and potentially accessible memories against which to assess functional recovery over an extended period of testing.

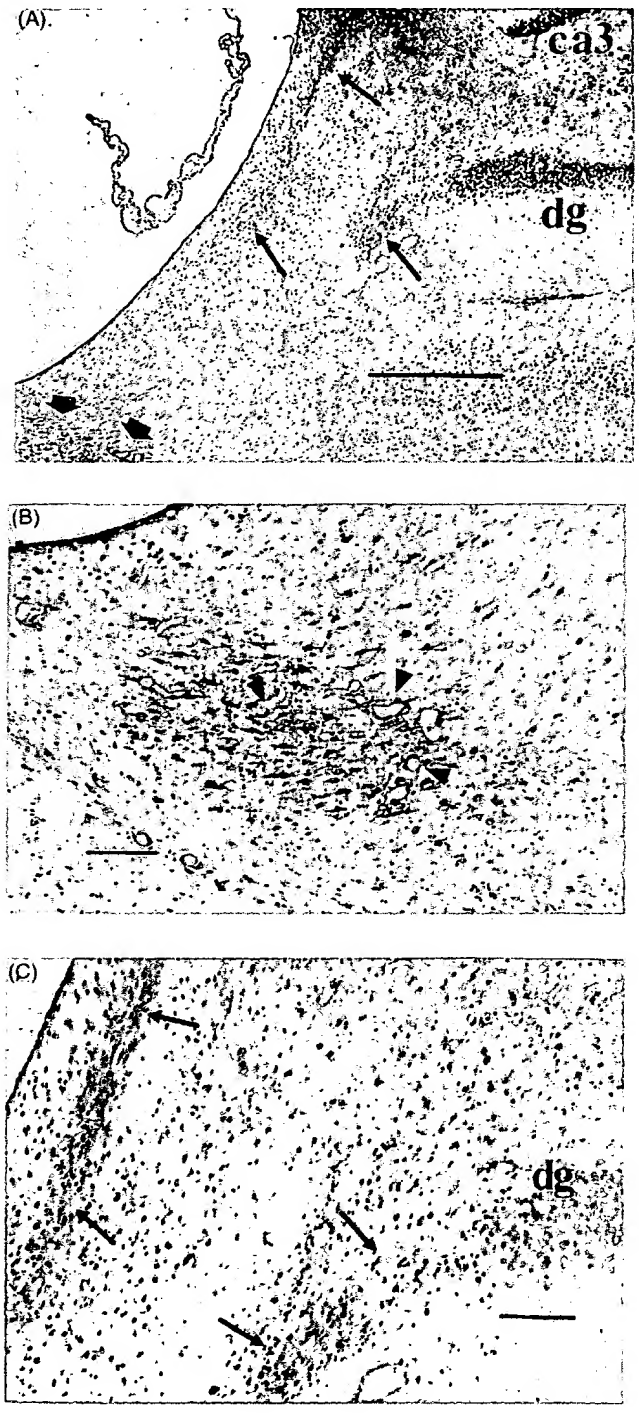
Foetal graft effects

The present results provide clear evidence of improvement both in the recall and learning of CDs following grafts of foetal tissue within the lesion sites. These results replicate the findings of Ridley and colleagues (Ridley *et al.*, 1997) in several respects. First, recall of discriminations was very substantially improved after foetal grafts in both studies. Secondly, learning of the first new CD task was only partially improved, hence graft-induced functional recovery appeared to be modest at this point. However, the present results show that if the animals are required to learn further discriminations, performance of marmosets with foetal grafts improves to control level and substantial recovery is evident. The failure of grafts to exert marked effects on cognitive performance when first tested could reflect incomplete integration of grafted cells into the host neural network and a longer interval may therefore be required for the development of full anatomical connectivity. Alternatively, grafted animals may require exposure to testing in order for functional integration to be forged by experience. In comparison, Mayer and colleagues found that rats with striatal grafts in unilateral striatal lesion sites required 2–3 weeks training before their reaction time to a contralateral light flash was normalized (Mayer *et al.*, 1992). Comparison of groups with and without prior experience would be necessary to elucidate whether time, training or these factors combined are critically responsible for recovery. Since impairment of lesioned animals was stable, it would also be possible to test animals early after transplantation to follow the time-course of recovery and to see whether this relates to the growth and connectivity of grafted cells. Within the small group of animals in the present experiment, there was no evidence that the eventual extent of functional recovery was related to specific features of the grafts, such as site, graft volume or number of transplanted cells, because all grafted animals showed maximal recovery. Grafts projecting haphazardly into the ventricles appeared to be just as effective as those which remained in the lesioned hippocampus. Anterior as opposed to posterior siting of grafts conferred no particular advantage. However, since bilateral CA1 lesions appear to be necessary to induce deficits in CD tasks (Ridley *et al.*, 1996), unilateral grafts might even be functionally effective in animals with bilateral lesions, and therefore correlations between graft

efficacy and number of grafted cells or graft volume would be difficult to establish.

MHP36 graft effects

The performance of marmosets with grafts of MHP36 clonal cells mirrored that of animals with foetal grafts throughout this experiment. Thus, marmosets with MHP36 grafts showed immediate improvement in recall of CDs, with partial improvement in learning the first new CD task, followed by full recovery to control level on subsequent learning of CD tasks 2–4. However, animals with foetal grafts were first tested 12 weeks after transplantation, whereas those with MHP36 grafts were tested after only 6 weeks. This choice of time-points reflects previous studies of graft development in rodents, in which foetal hippocampal grafts in ischaemic-lesioned CA1 have been shown to establish connectivity by 8–10 weeks (Mudrick and Baimbridge, 1991) and MHP36 grafts have been shown to migrate to the area of ischaemic CA1 cell loss after 4 weeks (Sinden *et al.*, 1997). The similar functional recovery despite the discrepancy between intervals before testing suggests, however, that the passage of time may not be as important for graft efficacy as previous experience of the animals which enables them to 'learn to use' their grafts (Mayer *et al.*, 1992). Effects of MHP36 grafts in CA1-lesioned marmosets resemble those in rats with ischaemic (four vessel occlusion) damage to the CA1 field of the hippocampus, in which both foetal and MHP36 grafts improved spatial learning and spatial working memory to control level. However, given the derivation of MHP36 cells from transgenic mouse neuroepithelial stem cells, the functional efficacy of these cells in primate as well as rodent species breaks new ground and suggests that neuroepithelial stem cells possess remarkable plasticity and low immune response provocation.



Graft histology

Histological examination indicated that foetal and MHP36 grafts exhibited very different patterns of host colonization. Foetal grafts were of two types. They either formed lobules attached to the ventricular margins of the CA1 field or they formed dense clusters within the hippocampus. No grafted foetal cell clusters were seen outside the hippocampus and lateral ventricles. These cell masses are typical of foetal grafts in rat hippocampus, although in the rat brain the corpus callosum above the CA1 field attracts more vigorous growth of foetal cells than the denervated CA1 field beneath (Hodges *et al.*, 1996). MHP36 grafts differed markedly from foetal grafts. Around the injection sites they formed diffuse

Fig. 8 Coronal section through the CA1 lesion site stained for reactivity to β-galactosidase. (**A**) MHP36 cells migrating from a ventral injection site (bottom left, thick arrows) upwards through the CA1 field towards the CA3 junction. This region also stained positive for βIII-tubulin, suggesting the presence of neurons within the graft (scale bar = 500 μm). At higher magnification (×100) the injection site (**B**) is well vascularized (arrowheads) and β-galactosidase positive cells appear more integrated than grafted CA1 cells (cf. Figs 6 and 7), and can be seen migrating upwards. Most of the cells near the injection site have an astrocytic appearance, but some show a neuronal phenotype. (**C**) MHP36 cells migrating towards the dorsal hippocampus in two broad streams: within the pyramidal cell layer heading towards CA3 and within the molecular layer heading towards the dentate gyrus (dg) which at this anterior level (~7 mm before the inter-aural line) is only just becoming visible. (Scale bars for **B** and **C** = 100 μm.)

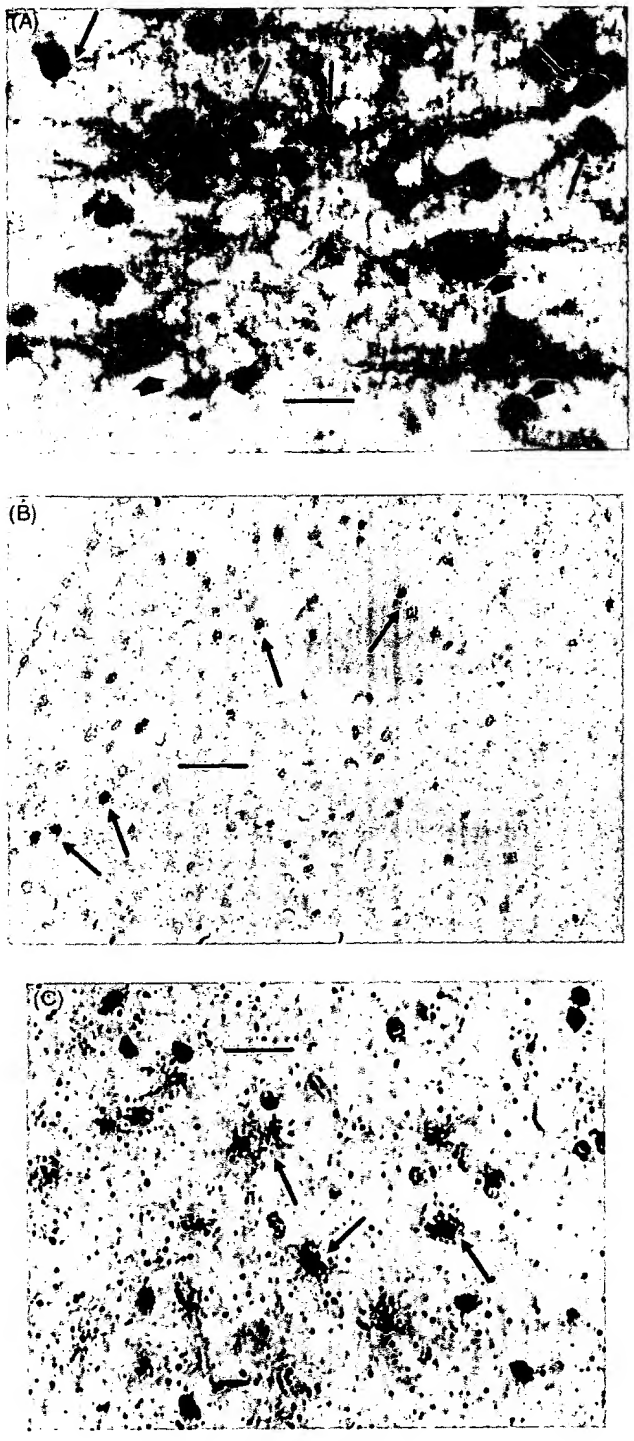


Fig. 9 (A) High power (×1000, scale bar = 10 μm) magnification of β-galactosidase positive MHP36 cells at the border of the CA1 field and the temporal white matter. Some cells appear astrocytic (arrows) whilst others resemble pyramidal neurons (thick arrows), with the neuron-like cells in the CA1 field and astrocytic cells moving into the white matter. (**B**) Autoradiograph of [^{3}H]thymidine labelled MHP36 cells show their relatively even and sparse distribution in the lesioned CA1 field, which contrasts with the clustering (cf. Fig. 6) of foetal CA1 cells (scale bar = 100 μm). (**C**) Double labelling of cells with β-galactosidase and [^{3}H]thymidine positive cells (arrows) confirms that these markers identify grafted MHP36 cells (scale bar = 10 μm).

aggregates which were more integrated into the host brain than the compact masses formed by foetal grafts. Unlike foetal cells, which remained in discrete clumps, MHP36 cells migrated away from the site of transplantation and were distributed throughout the lesioned CA1 field. This pattern of migration was surprisingly similar to that shown by MHP36 cells in ischaemic rat brain. In the rat, MHP36 cells migrate to the damaged CA1 layer where, to a lesser or greater extent, they form a compact and dense alignment of pyramidal-like cells, resembling the normal CA1 field (Sinden *et al.*, 1997). In marmosets the CA1 field is broader and less densely packed, which is characteristic of primate brain. However, with their even and widespread distribution MHP36 cells appeared to reconstruct the normal appearance of this field, as in the rat. MHP36 cells have been seen to migrate into the rat dentate granule layer damaged by needle penetration, just as they were found in one marmoset with dentate gyrus damage. As in the rat, MHP36 cells in the CA1 field showed both neuronal pyramidal-like and astrocytic types. Findings that MHP36 cells in white matter were astrocytic, whereas those in the dentate gyrus were neuronal and of granular appearance, support the evidence from other laboratories that differentiation of precursor stem cells is site-specific (Gage *et al.*, 1995; Shihabuddin *et al.*, 1996). However, our finding that MHP36 cells differentiated within discrete regions of brain damage contrasts with the findings of Gage and colleagues with FGF-responsive hippocampal progenitor cells (Gage *et al.*, 1995), and Shihabuddin and colleagues with the RN33B cell line cloned from medullary raphe progenitor cells. They found that grafts derived from stem cells develop host-type neuronal morphologies only when transplanted into unlesioned sites and not, as we found, lesioned sites (Shihabuddin *et al.*, 1996).

Conclusions

In conclusion, the present findings confirm that NMDA lesions of the marmoset hippocampal CA1 field induce long-lasting and profound impairment in retention of CDs first learned before lesioning, and in acquisition of new discriminations after lesioning, but have negligible effects on recall of SDs. These results suggest that lesions induced specific cognitive, but not motor, perceptual or motivational impairment. Grafts of foetal CA1 tissue and of conditionally immortalized clonal MHP36 cells derived from the H-2K^b-tsA58 transgenic mouse neuroepithelium were equally effective in reversing lesion-induced deficits. Recall of CDs was improved to control level on first testing after transplantation. Impairment of grafted animals in learning the first, but not subsequent CDs suggested that time and/or training may be important for functional graft recruitment. Gross histological examination indicated different patterns of innervation with foetal and MHP36 grafts. Foetal grafts formed lobules attached to the ventricular margin of the CA1 field, or clumps within the CA1 lesion sites. Grafted MHP36 cells were evenly distributed in the denervated host CA1 field

where they adopted both neuronal and astrocytic phenotypes. These results suggest that both foetal and MHP36 grafts promote recovery from damage to the hippocampus, and that this may involve partial reconstruction of hippocampal circuitry. However, in view of the very different patterns of foetal and MHP36 cell distribution, the precise mechanisms by which foetal and MHP36 grafts exert their functional effects may differ; further work is needed to determine factors which govern patterns of migration and to characterize grafted MHP36 cell types. Nevertheless, this first demonstration of robust improvement with a conditionally immortalized cell line in a primate species offers a promising prospect for the development of non-foetal grafts to alleviate the effects of circumscribed brain damage.

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